Potential roles of gut microbiome and metabolites in modulating ALS in mice

Potential roles of gut microbiome & metabolites in modulation of murine ALS

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Summary

Amyotrophic Lateral Sclerosis (ALS) is a genetically-driven neurodegenerative disorder, whose clinical manifestations may be influenced by unknown environmental factors. We demonstrate that ALS-prone SOD1-Tg mice feature a pre-symptomatic, vivarium-dependent dysbiosis and altered metabolite configuration, coupled with an exacerbated disease in germ-free or wide-spectrum antibiotic treatment conditions. We correlate 11 distinct commensals at our vivarium with mouse-ALS severity, and exemplify by their individual supplementation into antibiotic-treated SOD1-Tg mice that *Akkermansia muciniphila* (AM) ameliorates & *Ruminococcus torques* & *Parabacteroides distasonis* exacerbate mouse-ALS symptoms. Furthermore, AM-administered SOD1-Tg mice feature a CNS accumulation of AM-associated nicotinamide, which improves, upon systemic supplementation, motor symptoms and spinal-cord gene expression patterns in SOD-1-Tg mice. In humans, we identify distinct microbiome/metabolite configurations, including impaired systemic & cerebrospinal-fluid nicotinamide levels, in a small preliminary study assessing ALS patients versus household-controls. Together, we suggest that environmentally-driven microbiome-brain interactions may modulate murine ALS, and call for similar investigations in human ALS.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized by premature death of motor neurons and an average survival rate of 3-5 years from diagnosis[1]. Extensive efforts are being made to develop ALS-specific drugs, with only riluzole[2] and edaravone[3] showing modest efficacy. Environmental factors have been postulated to modify ALS disease course[4], and may include circulating small molecular-weight metabolites originating from the gastrointestinal (GI) tract and permeating the brain-blood barrier (BBB), where they can modulate metabolic, transcriptional and epigenetic programs in neurons and in other resident cells[4]. However, the causative role of such environmental factors in ALS or other CNS pathologies is only beginning to be unraveled[5].

The gut microbiome is an putative source of these potentially CNS disease-modifying bioactive metabolites, and has been recently suggested to contribute to the pathogenesis of neurological disorders[6,7] by impacting neuronal transmission, synaptic plasticity, myelination and complex host behaviors[8–10]. Several observations suggest that host-gut microbiome interface may be altered in murine ALS models[8–10], including an impaired gut barrier function and a dysbiotic microbiome configuration which was partially corrected by butyrate supplementation[11]. Stool 16S rDNA analysis of ALS patients yielded conflicting results, with one study noting a dysbiosis in 6 ALS patients compared to 5 healthy controls[12], while another showing no significant differences between 25 ALS patients and 32 healthy controls[13]. No direct functional microbiome investigation has been performed to date in this setting. In this study, we utilized mice and a preliminary human study in functionally assessing potential modulatory microbiome involvement in ALS.

Results

An altered gut microbiome exacerbates murine ALS symptoms

To assess the role of the gut microbiome in ALS we used the mSOD1 G93A (herein "SOD1-Tg") ALS mouse model. First, we depleted the microbiome of male and female SOD1-Tg or littermate controls by administrating broad-spectrum antibiotics (Abx) at the age of 40 days **(Extended Data Fig. 1a)**. Motor abilities were quantified using the rotarod locomotor test[14], hanging-wire grip test[15] and neurological

scoring[16]. Throughout the project, key repeat experiments were independently scored by two blinded researchers. Surprisingly, Abx treatment was associated with a significant and substantial exacerbation of motor abnormalities throughout ALS progression, compared to the water-treated SOD1-Tg group. Both the pooled results (N=15-30 mice per group, **Fig. 1a-c**) and independent results of each of the repeats ((N= 5-10 mice in each group of each repeat, three independent repeats, **Supporting Information Fig. 1**) demonstrated worsened results in the rotarod locomotor test **(Fig. 1a, Supporting Information Fig. 1a, 1d and 1g)**, the hanging-wire grip test **(Fig. 1b, Supporting Information Fig. 1b, 1e and 1h)** and neurological scoring **(Fig. 1c, Supporting Information Fig. 1c, 1f and 1i)**. Notably, Abx treatment did not affect rotarod or grip test performances in WT littermate controls at our vivarium, as compared to non-Abx-treated WT mice (**Fig. 1a-c and Supporting Information Fig. 1a-i**). A linear regression analysis further supported the negative effect of Abx treatment on these neuropathological measurements **(Extended Data Fig. 1b-d)**.

In agreement with these findings, spinal cord histopathological analysis (using luxol fast-blue staining) at day 140 revealed a significant reduction in motor neuron cell counts in Abx-treated compared to water-treated SOD1-Tg mice **(Extended Data Fig. 1e-f),** suggesting an increased motor neuron cell-death following chronic Abx exposure. Moreover, T_2-weighted magnetic resonance imaging (MRI) of the murine brain stem in areas known to degenerate in the SOD1-Tg model[17] **(Extended Data Fig. 1g)** demonstrated a prolonged T_2 relaxation time Abx-treated SOD1-Tg mice **(Extended Data Fig. 1h-o)**, indicative of higher levels of free water, enhanced brain atrophy and neurodegeneration[18]. Automated home-cage locomotion system revealed a significant reduction (p=0.03) in the activity of Abx-treated SOD1-Tg mice on day 100 compared to water-treated SOD1-Tg controls **(Extended Data Fig. 1p).** Abx-induced aggravation in motor function of SOD1-Tg mice was not associated with alterations of the main immune cell sub-populations in spinal cord, small intestine or colon lamina propria, compared to water-treated SOD1-Tg mice **(Supporting Information Fig. 1j-o)**, suggesting that the Abx-associated phenotypic differences were not immune-mediated.

Importantly, rederivation attempts of SOD1-Tg mice into the germ-free setting were associated with high-mortality rates of SOD1-Tg but not of WT littermate controls (failed rederivation attempts of 30 pregnant dams over a period of 18 months). Once rederivation succeeded, GF SOD1-Tg mice featured significantly enhanced mortality as compared to GF WT littermates or to colonized SOD1-Tg mice (**Fig. 1d**, pooled results, N=9-22 mice per group, **Supporting Information Fig. 1p-q,** two independent repeats, N=5-13 per group), which persisted even when GF mice were spontaneously colonized at day 115, suggesting that microbial drivers impact ALS progression at an earlier disease stage. Collectively, these results indicated a potential detrimental effect of Abx-mediated microbiome alteration (or its absence in GF mice) at our vivarium, on ALS manifestation in SOD1-Tg mice, suggesting that a locally dysbiotic gut microbiome configuration may modulate disease progression in this model.

SOD1-Tg mice develop a vivarium-dependent pre-clinical dysbiosis

These suggested microbial-mediated effects on ALS neuropathology presented an opportunity to identify locally-prevalent commensal strains potentially modulating ALS course. Indeed, assessment of fecal microbiome composition and function by 16s rDNA sequencing in SOD1-Tg and WT littermate controls at our facility indicated an early and significant microbiome compositional difference that persisted during disease course **(Fig. 2a-c, Extended Data Fig. 2a-m)**. Moreover, the total number of observed genera (alpha diversity) was higher in the SOD1-Tg stool at all time-points **(Extended Data Fig. 2n)**. However, total

fecal bacterial load did not vary between SOD1-Tg and WT controls **(Extended Data Fig. 2o)**. Moreover, even the gut microbiome configuration of Abx-treated SOD1-Tg and their WT littermate controls at our vivarium yielded significantly differential microbiome compositions (**Supporting Information Fig. 2**) Importantly, spontaneous colonization of GF SOD1-Tg and WT littermates at our vivarium resulted in de-novo dysbiosis (**Extended Data Fig. 3**), while these facility-dependent dysbiotic differences were not observed in a second non-barrier (non-SPF) vivarium featuring a near-absence of Akkermansia, Parabacteroides, Erysipelotrichaceae and Helicobacteraceae (**Supporting Information Fig. 3 and Supporting table 1**). Overall, these facility-dependent changes suggested that a combination of murine-ALS genetic susceptibility, coupled with a locally-prevalent commensal signature drive early pre-clinical dysbiosis possibly contributing to ALS modulation.

Likewise, when measured by fecal shotgun metagenomic sequencing, significant differences were noted in the microbiome composition of SOD1-Tg mice compared with littermate controls **(Fig. 2d and Extended Data Fig. 3a-b, Extended Data Fig. 4a-n)**. Functionally, SOD1-Tg and WT fecal bacterial metagenomes clustered separately with respect to microbial genes **(**for PC1: day 40, p=0.0002, day 60, p=0.0002, day 80, p=0.0005, day 100, p=0.0005, KEGG orthology, KO, **Fig. 2e)**, including a marked reduction in representation of genes encoding enzymes participating in tryptophan metabolism **(Extended Data Fig. 4o-p)** and substantial alterations in those of nicotinamide and nicotinate metabolism **(Extended Data Fig. 4q)**. A detailed metabolic assessment at the pre-clinical day 60 found no significant changes in food and water intake, respiratory exchange ratio, oxygen consumption, locomotion, and heat production **(Supporting Information Fig. 4)**.

Collectively, these results demonstrated that single-genotype-housed SOD1-Tg mice diverge in their gut microbial composition and function from their WT littermate configuration at our vivarium, even before the appearance of motor neuron dysfunction.

Commensal microbe contribution to ALS exacerbation

To determine whether these ALS-associated microbiome changes feature a causal contribution to disease features, we tested 11 strains, including *Eggerthella lenta, Coprobacillus cateniformis, Parabacteroides goldsteinii, Lactobacillus murinus, Parabacteroides distasonis, Lactobacillus gasseri, Prevotella melaninogenica, Eisenbergiella tayi* (member of the Lachnospiraceae family), *Subdoligranulum variabile, Ruminococcus torques and Akkermansia muciniphila,* all suggested by our composite 16S rDNA and shotgun metagenomic analysis to be correlated with severity of ALS progression in the SOD1-Tg model at our vivarium (**Extended Data Fig. 2** and **4**). We mono-inoculated anaerobic cultures of each of the above strains (stationary phase O.D.=0.4-0.7) into Abx pre-treated SOD1-Tg and WT mice, by repeated oral administration at 6 day-intervals for a total of 15 treatments. Most of the indicated bacteria did not affect ALS symptoms **(Extended Data Fig. 5)**. Supplementation of Abx-treated SOD1-Tg mice with *Parabacteroides distasonis* (PD, **Extended Data Fig. 5**) and *Ruminococcus torques* (RT, **Extended Data Fig. 6 and Supporting Information Fig. 5** for pooled and 4 independent repeats) exacerbated disease progression, while *Lactobacillus gasseri* and *Prevotella melaninogenica* treatments (LG and PM, respectively) showed disease-promoting effects in some, but not all, of the behavioral tests (**Extended Data Fig. 5**). Of note, none of the tested 11 bacterial strains affected motor abilities in WT animals (**Extended Data Fig. 5g-l** for 9 tested bacterial strains, and **Extended Data Fig. 6 and Supporting Information Fig. 5** for RT) and all resulted in a distinct microbiome composition in SOD1-Tg mice

(**Supporting Information Fig. 6)**. Taken together, these results suggest that several commensals might contribute to motor neuron degeneration in the SOD1-Tg ALS mouse model.

AM colonization ameliorates murine ALS and prolongs survival

One of the differentially altered species in SOD1-Tg mice at our vivarium was *Akkermansia muciniphila* (AM), with both 16S rDNA **(Extended Data Fig. 2c)** and shotgun metagenomic sequencing **(Extended Data Fig. 4b** and **Fig. 3a)** demonstrating that it gradually reduced in its abundance as disease progressed in SOD1-Tg mice, as compared to the WT littermate microbiome. Time-dependent AM reduction in SOD1-Tg mice was validated using AM-specific qPCR **(Fig. 3b)**. In contrast to all of the other tested strains, treatment of Abx pre-treated SOD1-Tg and WT mice with an anaerobically mono-cultured AM strain (BAA-835, O.D. =0.7, stationary phase), was associated with improved motor function in AM-treated SOD1-Tg mice as quantified by the rotarod, grip and neurological scoring tests and assessed in pooled samples (N=34-62 mice per group, **Fig. 3c-e**) or independently from 6 repeats **(**N= 5-25 mice in each group of each repeat, **Extended Data Fig. 5a-c and Supporting Information Fig. 7).** This AM-mediated functional improvement was accompanied by a higher motor neuron survival in the AM-treated SOD1-Tg spinal cords, as compared to vehicle-treated Abx-pre-treated SOD1-Tg mice **(Extended Data Fig. 7a-b,** p=0.0041**)**. Importantly, AM treatment significantly and substantially prolonged the life-span of SOD1-Tg mice compared to vehicle-treated mice or to SOD1-Tg mice treated with other commensal microbiome species serving as bacterial controls **(Fig. 3f)**. AM treatment reduced brain atrophy at day 140, as indicated by MRI **(Extended Data Fig. 7c-f)**. No differences in gut barrier function, as measured by systemic FITC-dextran influx, were found at day 120 between AM-, PBS- and other microbial treated SOD1-Tg and WT mice **(Extended Data Fig. 7g)**. The microbiome metagenome of AM-treated SOD1-Tg mice clustered differently than that of PBS-treated SOD1-Tg controls **(Extended Data Fig. 7h)**. As expected, AM relative abundance was significantly increased in stool samples of AM-treated as compared to vehicle-treated SOD1-Tg mice **(Extended Data Fig. 7i)**. In contrast, WT mice harboring high and stable indigenous AM levels at our vivarium featured competitive exclusion of exogenously-administered AM whose levels rose only upon prolonged administration (**Extended Data Fig. 7j)**. Moreover, AM was found to colonize more broadly and efficiently in different regions of the SOD1-Tg GI tract comparing to the WT GI tract (**Extended Data Fig. 7k-l)**. Consequently, AM supplementation following Abx treatment altered the microbiome composition of both WT and SOD1-Tg mice in distinct manners **(Extended Data Fig. 7m-n)**. Similarly, mono-colonization of Abx-pretreated SOD1-Tg and WT littermates with another strain of AM (ATCC 2869) induced a significant improvement in motor abilities **(Extended Data Fig. 7o-q)**. Histopathological analysis of distal colon mucus of AM- or PBS-treated SOD1-Tg at day 140 demonstrated an intact inner mucus layer mucus in both AM supplemented and PBS-treated SOD1-Tg mice (**Extended Data Fig. 8a)**, with only AM-treated SOD1-Tg mice featuring penetrating bacteria in the inner mucus and in rarely in the crypts (**Extended Data Fig. 8b, white arrows)**. A proteomic analysis did not feature significant differences in mucus components levels in AM-supplemented mice (**Extended Data Fig. 8c-j).** Collectively, assessment of multiple differentially expressed gut commensals by their mono-inoculation into SOD1-Tg mice identified selected commensals that adversely (PD, RT, and potentially LG and PM) or favorably (AM) modulate murine-ALS disease course and severity.

AM attenuates murine ALS by systemically elevating Nicotinamide levels

Given the remoteness of the gut microbiome from the CNS disease site, we hypothesized that a potential systemic influx of microbiome-regulated metabolites may impact motor neuron susceptibility in SOD1-Tg mice by translocating to the CNS[19,20]. To this aim, we utilized untargeted metabolomic profiling to identify candidate microbiome-dependent molecules differentially abundant in sera of AM-supplemented and vehicle controls, during the early stage of ALS (day 100). Out of 711 serum metabolites identified in SOD1-Tg mice, 84 metabolites were significantly altered by AM supplementation, out of which 51 were elevated by AM treatment (**Fig. 4a and Extended Data Fig. 9a-c)**. Of these, the biosynthetic genes (nucleotide sequences, KEGG database) of only 6 metabolites were aligned to our metagenomic index, with two metabolites, Nicotinamide and Phenol sulfate, featuring the highest metagenomic probabilities to be synthesized by the WT microbiome over the SOD1-Tg microbiome at our vivarium (**Extended Data Fig. 9d**). Administration of Phenol sulfate to SOD1-Tg mice, using subcutaneously implanted slow-release mini osmotic pumps did not affect ALS symptoms *in vivo* (**Extended Data Fig. 9e-g**).

We next focused on NAM, given marked alterations in the metagenomic NAM biosynthetic pathway between SOD1-Tg and WT controls (**Fig. Extended Data Fig. 4q),** enrichment in serum levels of NAM biosynthetic intermediates upon AM supplementation (**Fig. 4b**), reduced abundance of genes of the gut microbiome-derived tryptophan metabolizing pathway which may be involved in generation of NAM[21] in naïve SOD1-Tg mice **(Fig. Extended Data Fig. 4o-p)**, and altered metabolites of the tryptophan pathway upon Abx treatment or AM supplementation **(Extended Data Fig. 9h-i)**. We first measured NAM levels in anaerobically-grown AM and control commensal isolates, using targeted metabolomics and found significantly higher levels of NAM in the medium of AM cultures, compared to heat-killed AM or from other commensal isolates **(Fig. 4c)**. *In vivo*, CSF NAM levels were significantly higher in both AM-treated SOD1-Tg and WT mice already at age 100 days (early-stage disease, **Fig. 4d)**. During advanced stages of the disease (day 140), CSF NAM levels were significantly higher in AM-treated SOD1-Tg mice but not in AM-treated WT mice as compared to untreated controls **(Extended Data Fig. 9j)**, potentially reflecting gut colonization stability differences between WT and SOD1-Tg mice (**Extended Data Fig. 7i-n**). Importantly, 8 out of the 10 AM genome-related genes that encode enzymes participating in NAM metabolism, were significantly enriched in AM-treated SOD1-Tg mice compared to vehicle-treated SOD1-Tg mice **(Extended Data Fig. 9a and Supporting Information table 2)**, indicating that AM supplementation in SOD1-Tg mice may directly modify functional NAM biosynthesis.

To causally link increased systemic NAM levels to the associated phenotypic effects noted upon AM supplementation, we continuously supplemented SOD1-Tg mice with NAM, administered subcutaneously through implanted mini-osmotic pumps. Indeed, NAM levels were significantly increased in the CSF **(Fig 4e)** and sera (**Extended Data Fig. 10a)** of NAM-treated SOD1-Tg mice compared to water-treated controls. Importantly, NAM-treated SOD1-Tg mice performed significantly better than vehicle-treated SOD1-Tg mice, in both behavioral and neurological motor tests, as indicated by a pooled analysis **(**N=30 mice per group**, Fig. 4f-h)** or independently in three repeats **(**N=10 mice in each group of each repeat, **Supporting Information Fig. 8a-i)**. Of note, NAM treatment resulted in a non-significantly trend to improve survival **(Extended Data Fig. 10b)**, possibly reflecting insufficient dosing or exposure time, or the necessity for integration of other AM-mediated modulatory mechanisms (**Fig. 3g**) in reaching the observed AM-induced survival benefit. We further inoculated Abx-pretreated SOD1-Tg mice with either WT *E. coli* as control or with the ΔnadA *E. coli* compromised in NAM production (**Extended Data Fig. 10c**). Of note, *E. coli* is considered a poor colonizer of the mouse GI tract[22]. While ΔnadA *E. coli* supplementation did not affect

rotarod and grip test performances (**Extended Data Figure 10d-e** and **Supporting Information Fig. 9**), it significantly exacerbated the neurological scores of SOD1-Tg mice compared to the WT *E. coli*-treated animals **(Extended Data Fig. 10f and Supporting Information Fig. 9)**, suggesting that NAM secreted from gut bacteria, even with poor colonization capacity, is able to impact some motor abilities in this ALS model.

Potential AM and NAM mechanisms of ALS modulation

To explore potential downstream mechanisms by which AM and NAM may support motor neuron survival and ameliorate ALS progression in SOD1-Tg mice, we conducted bulk RNA-sequencing (RNA-seq) of spinal cord samples collected from AM- and NAM-treated mice. Overall, false discovery rate (FDR)-corrected expression of 213 genes significantly changed following NAM treatment of SOD1-Tg mice **(Extended Data Fig. 11a)**. 31 of these genes also significantly correlated in their expression pattern following AM treatment **(Extended Data Fig. 11b)**. Annotating the NAM-responsive genes to phenotype ontology resulted in a significant 21% fit to 4 categories related to abnormal brain morphology, physiology and movement **(Extended Data Fig. 11c)**. The most significantly enriched GO (Gene Ontology) pathways between AM and NAM interventions **(Extended Data Fig. 11d-e)** were related to mitochondrial structure and function, Nicotinamide adenine dinucleotide^{+} (NAD^{+}) homeostasis and removal of superoxide radicals, canonical functions known to be disrupted in ALS. Interestingly, 28.6% of the shared genes between AM and NAM treatments were found to be regulated by the transcription factor Nuclear Respiratory Factor-1 (NRF-1, **Extended Data Fig. 12a**), known to control mitochondrial biogenesis, electron transport chain activity and oxidative stress[23–27]. Potential NRF-1 contribution to this modulatory effect merits further studies. Notably, NAM and AM treatments did not alter spinal cord *Slc1a2* expression levels (**Extended Data Fig. 12c-e**) encoding to the excitatory amino acid transporter 2 (EAAT2) in astrocytes, suggesting that the effect may not stem from NAM-associated glutamate excitotoxicity.

Dysbiosis & impaired NAM levels in human ALS patients

Finally, we examined preliminary links between the SOD1-Tg findings at our vivarium and features of human ALS. To this aim, we performed a small-scale human observational study, by collecting stool samples from 37 ALS patients and 29 healthy BMI- and aged-matched family members as controls and sequencing their gut microbiome metagenomes. The microbiome composition of ALS patients, as quantified by shotgun metagenomic sequencing, was significantly different to that of healthy control household members (**Fig. 5a**, for PC1: $p = 1.3\text{x}10^{-5}$). We observed only marginally significant difference in specific bacterial species abundances after FDR correction **(Extended Data Fig. 13a)**, with five bacterial species reaching nearly-significant for ALS microbiome ($q < 0.1$), out of which one featured significant correlation with serum NAM levels **(Supporting Information table 3 and Extended Data Fig. 13b).** Functionally, ALS microbiomes showed a significant difference in the global bacterial gene content **(Fig. 5b,** for PC1: $p = 2.55\text{x}10^{-10}$), accompanied by FDR-corrected (adjusted for these pathways) decrease in several key genes participating in tryptophan and in NAM metabolism **(Extended Data Fig. 13c and Supporting Information table 4)**. Interestingly, several of these significantly reduced genes were mapped to the *A. muciniphila* genome, suggesting potential AM involvement that merits larger future studies.

Untargeted metabolomic profiling of ALS patient sera revealed multiple significantly-changed metabolites, including elevated riluzole (an ALS exogenously-administrated treatment), creatine and 3-hydroxy-2-ethylpropionate and reduced methyl indole 3-acetate and triethanolamine **(Extended Data Fig. 13d**). Interestingly, key molecules of the tryptophan-nicotinamide metabolic pathway were

significantly altered in the sera of ALS patients, among them Indoleacetate, Kynurenine, Serotonin and circulating Nicotinamide **(Extended Data Fig. 13e)**, suggesting an aberrant NAM metabolism in some of these human ALS cases. Targeted serum metabolomics further validated the marked NAM decrease in the sera of 60 ALS patients compared with 33 healthy controls. **(Fig. 5c)**. Moreover, serum NAM levels from ALS patients mildly but significantly correlated both with human ALS Functional Rating Scale (FRS) scores **(Extended Data Fig. 13f)** and with the levels of the microbiome gene encoding for one of the rate-limiting NAM biosynthetic reactions **(Extended Data Fig. 13g)**. We further found the levels of NAM in the CSF of 14 ALS patients to be significantly lower than those of 17 healthy household controls, driven by some patients featuring markedly low NAM CSF levels **(Fig. 5d)**. The clinical implications of the above preliminary metabolomic findings and associations, and whether they apply to all or to subsets of ALS patients, merit further validation and exploration by human-focused prospective studies.

Discussion

Our findings highlight the potential cooperative activity of genetic risk and locally-varying environmental modulatory factors in impacting ALS, with the gut microbiome serving as a 'hub' relaying these environmental signals to the host, as was noted in other multifactorial disease such as IBD[29]. Of note, our study mainly focuses on one ALS animal model, in the context of one vivarium, and mechanistically exemplifies one or multiple differentially-expressed metabolites in ALS (NAM). Future studies may decipher complementary ALS-relevant CNS functions of other gut-derived commensals and metabolites identified by us and by others. Importantly, our human data is preliminary and observational, and is not aimed or sufficient to constitute a treatment recommendation of any sort in this devastating disease. Larger future prospective human ALS studies, including ones incorporating genetic disease variants, are needed to validate the suggested microbiome associations, in assessing causal impacts of potential microbiome modulators of human ALS. These limitations notwithstanding, our study suggests a potential modulatory involvement of the gut microbiome in ALS, that may help delineate some aspects of its elusive pathophysiology, while providing an opportunity to identify modifiable environmental and microbial therapeutic targets.

Data availability

The sequencing data has been deposited at the European Nucleotide Archive database with the accession number PRJEB32767.

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References referring to the methods section are depicted in the Supplmentary information

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Author contributions

E.B., S.B., and H.S. designed, performed and interpreted the experiments, and wrote the manuscript; S.B., D.R. performed and analyzed all the microbiome sequencing and analysis; E.B., S.B., H.S., and D.R. equally contributed to the study. U.M., Y.C., N.B., and I.L. assisted in computational analysis. M.D.B, C.K., C.M., Y.H., D.K., N.Z., N.A, I.B., Y.K., M.T. and L.Al. performed and supervised key animal experiments. T.M. & A.B. performed the metabolomics experiments. M.Zu., M.Za., R.B-Z performed and supervised the human experimentation. A.H. oversaw animal experimentation including in germ-free mice. M.S. and A.I. provided key help and insights on the project. L.Ar. performed proteomic studies, M.E.V.J. performed the colon immunohistology staining and G.C.H. supervised these studies. M.G., E.S. and E.E. conceived the study, supervised the participants, interpreted the experiments, and wrote the manuscript.

Competing interests

E.S. and E.E. are paid consultants at DayTwo and BiomX. None of this work is related to, shared with or licensed to these or any other commercial entity. None of the other authors have competing interests related to this work.

Figure legends

Figure 1. Antibiotic treatment exacerbates motor symptoms in an ALS mouse model.

Evaluation of motor symptoms by **(a)** rotarod, **(b)** hanging-wire grip tests and **(c)** neurological scoring. *P=0.0256, ***P=0.0007 and 0.0005, two-sided Mann-Whitney U test. The experiment was repeated 3 times, (N=20 SOD1-Tg water, 18 SOD1-Tg Abx, 14 WT water and 15 WT Abx mice). Error bars represent

mean ± S.E.M. **(e)** Survival of GF (N=14) and SPF (N=17) SOD1-Tg mice. **P<0.0027, two-sided Log-rank test. The experiment was repeated twice.

Figure 2. SOD1-Tg mice develop early gut microbiome compositional and functional changes. Weighted UniFrac PCoA on **(a)** day 40 (pre-symptomatic), **(b)** day 100 (disease onset) and **(c)** day 140 (advanced disease). The experiment was repeated 3 times, (N=6 mice in each group). **(d)** Species-level taxa summary obtained by gut microbiome metagenomic shotgun sequencing of WT and SOD1-Tg stool samples during disease progression. **(e)** PCA of KEGG entries of WT and SOD1-Tg microbiome. p=1.57x10^{-14}, Spearman correlation coefficient. N=6 WT mice for days 40, 60, 100 and SOD1-Tg mice for days 40 and 80, and N=5 WT mice for day 80 and SOD1-Tg mice for days 60 and 100.

Figure 3. *Akkermansia muciniphila* colonization ameliorates motor degeneration and increases life-span in SOD1-Tg mice. (a) Linear regression of AM relative abundance (16S rDNA sequencing) of SOD1-Tg and WT stool over time (Spearman correlation coefficient). **(b)** qPCR of AM 16S gene copies in fecal DNA extract N=5 WT and 6 SOD1-Tg mice, two-sided Mann-Whitney U test **P=0.0043. Error bars represent mean ± S.E.M. **(c)** Rotarod, **(d)** hanging-wire grip test and **(e)** neurological scoring. N=62 SOD1-Tg PBS, 61 SOD1-Tg AM, 37 WT PBS and 36 WT AM mice. two-sided Mann-Whitney U test. ***P=0.0002, ****P<0.0001. Error bars represent mean ± S.E.M. The experiment was repeated 6 times (N=5-26 mice). **(f)** Survival of PBS- (N=8), AM- (N=9), *Prevotella melaninogenica* (PM, N=4)- and *Lactobacillus gasseri* (LG, N=5)-treated mice ***P<0.0003, two-sided Log-rank test.

Figure 4. *Akkermansia muciniphila* treatment is associated with enhanced nicotinamide biosynthesis in SOD1-Tg mice. (a) Significantly increased serum metabolites in SOD1-Tg mice treated with AM (upper-right quadrant N=7 mice). Two-sided FDR corrected Mann Whitney U ranksum test. **(b)** Serum levels nicotinamide pathway metabolites in SOD1-Tg and WT mice treated with AM or PBS. **(c)** Nicotinamide levels in bacterial cultures. N=5 cultures in each group, **P<0.005, ***P<0.0005, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(d)** CSF nicotinamide levels of SOD1-Tg and WT mice treated with AM or PBS on day 100. **P=0.0041, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(e)** CSF NAM levels in NAM and vehicle treated SOD1-Tg mice (N=10 mice) *P=0.0232, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. Motor performances of NAM or vehicle treated SOD1-Tg mice using subcutaneous osmotic pumps indicated by **(f)** rotarod, **(g)** hanging-wire grip test and **(h)** neurological scoring. ****P<0.0001 two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. The experiment was repeated 3 times, (N=30 mice in each group).

Figure 5. Microbiome-derived nicotinamide metabolism is impaired in ALS patients (a) PCA of bacterial species composition (for PC1 p=1.3x10^{-5}, Spearman correlation coefficient) or **(b)** KEGG orthology (KO) annotated bacterial genes (for PC1 p=2.55x10^{-10}, Spearman correlation coefficient) obtained by stool metagenomic sequencing from ALS patients (N=37) and healthy controls (family members, N=29). **(c)** Serum and **(d)** CSF NAM levels of ALS patients (N=60 for serum and 14 for CSF) and healthy controls (N=33 for serum and 17 for CSF), ****P<0.0001, ***P=0.0001, Mann Whitney U test. Error bars represent mean ± S.E.M.

Extended Data Figure 1. The effects of microbiome depletion on ALS symptoms in SOD1-Tg mice. (a) (a) Experimental design. Linear regression of motor functions over time in SOD1-Tg and WT treated indicated by **(b)** rotarod, **(c)** hanging-wire grip test, and **(d)** neurological score. N=20 SOD1-Tg water and Abx, 14 WT water and 15 WT Abx mice. **(e)** Histological images and **(f)** quantification of spinal cord motor neurons of 140-day old water- and Abx-treated SOD1-Tg mice. (N=10 SOD1-Tg water, 5 SOD1-Tg Abx),*P=0.028 , two-sided Mann-Whitney U test. The experiment was repeated twice. Error bars represent mean ± S.E.M. **(g)** MRI of murine ALS brain areas and their corresponding (f) T2 maps. The experiment was repeated twice. **(h-o)** quantification of T_2 relaxation time between water and Abx-treated SOD1-Tg mice throughout ALS. N=9 mice in each group. *P<0.05, **P<0.005, ****P<0.00005, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(p)** Home cage locomotion analysis over a period of 46 h, days 100–101 (N=5 mice). *P=0.03, two-sided Mann-Whitney U test.

Extended Data Figure 2. Microbial compositional dynamics in the SOD1-Tg mouse model across ALS progression. (a) Taxa summary of bacterial phyla and **(b)** genera of WT and SOD1-Tg mice obtained by 16S rDNA sequencing of stool samples. **(c)** Relative abundances of significant differentially representative genera between SOD1-Tg and WT mice. N=6 mice in each group, two-sided FDR-corrected Mann-Whitney U test. **(d-m)** FDR-corrected linear regression comparison of representative bacterial relative abundance change during ALS progression between WT and SOD1-Tg stool. Spearman correlation coefficient. N=6 SOD1-Tg and 4 WT mice. **(n)** Alpha diversity of SOD1-Tg and WT microbiomes. N=7 SOD1-Tg and 5 WT mice, *P<0.05, **P<0.005, ***P<0.0005, two-sided FDR-corrected Mann-Whitney U test. Error bars represent mean ± S.E.M. The experiment was repeated 3 times. **(o)** qPCR-based quantification of total 16S copy-number in 1 ng of DNA extracted from stool samples of SOD1-Tg and WT mice. N=6 mice, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M.

Extended Data Figure 3. Microbial spontaneous colonization in Ex-GF SOD1-Tg mouse model across ALS progression. (a) Taxa summary of bacterial genera in individual Ex-GF WT and SOD1-Tg undergoing spontaneous bacterial colonization during ALS course. **(b-e)** Weighted UniFrac PCoA of Ex-GF WT and SOD1-Tg mice on days 4, 5, 53 and 63 following spontaneous colonization. N=6 mice in each group, two-sided FDR-corrected Mann-Whitney U test **(f-i**) FDR corrected volcano plots of significantly enriched bacterial genera of Ex-GF WT and SOD1-Tg during ALS course on days 4, 5, 53 and 63 following spontaneous colonization. N=6 mice in each group. two-sided FDR-corrected Mann-Whitney U test.

Extended Data Figure 4. Metagenomic differences between WT and SOD1-Tg fecal microbiomes (a) PCoA plot of bacterial composition (N=23 SOD1-Tg and 24 WT mice), and **(b)** Taxa summary representation at the species level of gut microbiome of WT and SOD1-Tg mice obtained by metagenomic shotgun sequencing. The experiment was repeated twice (N=6 mice). **(c-n)** FDR-corrected linear regression comparison of representative bacterial relative abundance change during ALS progression between WT and SOD1-Tg stool. N=6 mice in each group, Spearman correlation coefficient. **(p)** Schematic representation and **(q)** heatmap of bacterial gene abundances of tryptophan metabolism. N=6 mice, two-sided FDR-corrected Mann-Whitney U test. *P<0.05, **P<0.005, ***P<0.0005. **(r)** Heatmap of bacterial gene abundances of the nicotinamide and nicotinate biosynthesis pathway. N=6 mice, *P<0.05, **P<0.005, ***P<0.0005, two-sided Mann-Whitney U test. FDR was used for multiple comparisons.

Extended Data Figure 5. Mono-colonization of Abx pre-treated SOD1-Tg mice with selected ALS-correlating microbiome strains. Motor functions of Abx pre-treated SOD1-Tg mice treated with PBS (N=7), *Eggerthella lenta* (EL, N=7), *Coprobacillus cateniformis* (CC, N=6), *Parabacteroides goldsteinii* (PG, N=6), *Lactobacillus murinus* (LM, N=8), *Parabacteroides distasonis* (PD, N=5), *Lactobacillus gasseri* (LG, N=8), *Prevotella melaninogenica* (PM, N=4), or *Akkermansia muciniphila* (AM, ATCC 835, N=6) indicated by **(a)** rotarod, **(b)** hanging-wire grip test and **(c)** neurological scoring. **(d-f)** Motor functions of Abx pre-treated SOD1-Tg mice treated with PBS or *Eisenbergiella tayi* (ET, N=6), or **(g-i)** Subdoligranulum variabile (SV, N=7). **(J-l)** Motor functions of Abx pre-treated WT littermate controls treated with PBS, LM, PD, LG, PM or AM. *P<0.05, **P<0.005, ***P<0.0005, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M.

Extended Data Figure 6. The effects of *Ruminococcus torques* mono-colonization on ALS progression in SOD1-Tg mice. (a) Linear regression of *Ruminococcus torques* (RT) relative abundance (16S rDNA sequencing) of SOD1-Tg and WT stool N=6 mice, Spearman coefficient. **(b)** Rotarod, **(c)** hanging-wire grip test and **(d)** neurological scoring of Abx-pretreated WT and SOD1-Tg treated with PBS or RT. N=18 SOD1-Tg PBS, 20 SOD1-Tg RT and 23 WT PBS and RT mice. *P=0.0205, **P=0.0029, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(e)** Histological images and **(f)** quantification of spinal cord motor neurons of 140 days old PBS- (N=5) and RT-treated SOD1-Tg (N=3) mice. Error bars represent mean ± S.E.M. The experiment was repeated 3 times. **(g)** Brain areas and their corresponding **(h-m)** T_2 relaxation time quantification between PBS and RT-treated SOD1-Tg mice throughout the disease. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005, two-sided Mann-Whitney U test. The experiment was repeated twice, N=5 mice in each group. Error bars represent mean ± S.E.M.

Extended Data Figure 7. The effects of *Akkermansia muciniphila* treatment on ALS manifestation and microbiome composition in SOD1-Tg mice. (a) Histological images and **(b)** spinal cord motor neuron quantification in 140-day old PBS- and AM-treated SOD1-Tg mice. N=7 mice in each group *P<0.0111, **P<0.0041, two-sided Mann-Whitney U test. The experiment was repeated twice. Error bars represent mean ± S.E.M. **(c-f)** T_2 relaxation time in PBS and AM (ATCC 835)-treated Abx-pretreated SOD1-Tg mice at days 100 and 140. N=5 mice in each group, ***P<0.0005, ****P<0.00001, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(g)** Systemic FITC-dextran measurement at 120 days WT and SOD1-Tg treated with PBS (N=3 and 7), AM (N=3 and 9), *P. Melaninogenica* (PM, N=5 and 4) or *L. gaseri* (LG, N=5). Two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(h)** PCoA of bacterial species compositions in SOD1-Tg mice treated with PBS (N=33) or AM (N=31). AM relative abundance in **(i)** SOD1-Tg or **(j)** WT mice treated with PBS or AM. N=8 mice, *P<0.05, ***P<0.0005, ****P<0.00005, two-sided Mann-Whitney ranksum test. Error bars represent mean ± S.E.M. **(k)** Individual and **(l)** averaged qPCR-based fold-change of *Akkermansia muciniphila* 16S copy number in mucosal and luminal samples across the GI tract of 140 days old AM or PBS treated WT and SOD1-Tg mice, N=5 mice in each group. **(m** Genera bacterial summary of SOD1-Tg or **(n)** WT mice treated with PBS or AM. Abx-pretreated SOD1-Tg and WT littermate control mice were treated orally with AM (ATCC 2869) or PBS as vehicle from age 60 days until the experimental end-point. On days 60, 80, 100, 120 and 140 motor performance of the mice was assessed by **(o)** rotarod, **(p)** hanging-wire grip test and **(q)** neurological scoring. (N=6 SOD1-Tg PBS, AM 2869 and WT AM 2869 and 7 WT PBS mice), **P=0.0022, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M.

Extended Data Figure 8. *Akkermansia muciniphila* treatment alters mucus properties of SOD1-Tg mice. Immunohistochemical assessment of distal colon mucosa of 140 days old **(a)** PBS- and **(b)** AM- (BAA-835) Abx-pretreated WT and SOD1-Tg mice. DNA stained with Sytox-green (green) and the mucus with an anti-MUC2C3 antiserum and goat anti-Ig (red). The non-stained areas between the epithelium and outer mucus/luminal bacteria is the inner mucus layer, allows bacteria identification. The experiment was performed once. Heatmap representation of **(c)** total mucus proteomic landscape and **(d)** AM-related peptides and **(e-j)** quantification of key representative mucus components. N=4 SOD1-Tg PBS and 8 SOD1-Tg AM mice, two-sided Mann-Whitney U test. P=N.S. Error bars represent mean ± S.E.M.

Extended Data Figure 9. Serum metabolomic profile is affected by antibiotics or AM treatment in ALS SOD1-Tg mice. Heatmap representation of serum metabolites of 100 days old **(a)** naïve SOD1-Tg and their WT littermates, **(b)** water or Abx-treated SOD1-Tg mice, **(c)** PBS or AM-treated SOD1-Tg mice. **(d)** Scoring of top six serum metabolites which significantly altered by Abx treatment in SOD1-Tg mice by their potential to originate of the gut microbiome. N=8 mice in each group. Motor performances of Phenol sulfate (N=8) or water treated (N=7) SOD1-Tg mice using subcutaneous osmotic pumps indicated by **(e)** rotarod, **(f)** hanging-wire grip test and **(g)** neurological scoring. Two-sided Mann-Whitney U test. P=N.S. Error bars represent mean ± S.E.M. Non-targeted metabolomics assessment of tryptophan metabolism of **(h)** water and Abx- treated or **(i)** PBS and AM-treated 100 days old SOD1-Tg mice. N=7 mice in each group. **(j)** CSF nicotinamide levels of SOD1-Tg and WT mice treated with AM or PBS on day 140. *P=0.0.205, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(k)** Schematic representation of the microbiome-derived nicotinamide producing genes in AM treated SOD1-Tg fecal samples.

Extended Data Figure 10. Nicotinamide ameliorates ALS progression in SOD1-Tg mice. (a) sera NAM levels in NAM and vehicle treated SOD1-Tg mice (N=10 mice) *P=0.0433, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. **(b)** Survival assessment of NAM and vehicle treated SOD1-Tg mice p=0.1757. N=10 mice in each group, two-sided Log-rank test. **(c)** Nicotinamide levels in WT or ΔnadA *E. coli* cultures (N=5). **P=0.0079, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M. Motor performances of WT or ΔnadA *E. coli*-inoculated Abx-pretreated SOD1-Tg mice indicated by **(d)** rotarod, **(e)** hanging-wire grip test and their **(f)** neurological scores. N=18 WT-*E.coli* and 16 ΔnadA *E. coli* SOD1-Tg mice. ***P=0.0007, two-sided Mann-Whitney U test. Error bars represent mean ± S.E.M.

Extended Data Figure 11. Uncovering potential downstream motor neuron modulatory mechanisms of AM and NAM treatments. (a) Heatmap of FDR-corrected differentially-expressed genes in the spinal cords of NAM-treated SOD1-Tg mice (N=10 mice), two-sided FDR corrected Mann Whitney U ranksum test. **(b)** Spearman correlation of spinal cord transcripts log2 fold change between AM- and NAM-treated SOD1-Tg mice, P<0.0001, (N=10 mice) **(c)** Comparison of the significantly differentially-expressed genes following NAM treatment with the KOG database classified into 4 neuropathological groups. FDR-corrected gene set enrichment distribution of spinal cord transcripts of **(d)** NAM-treated and **(e)** AM-treated SOD1-Tg mice into biological process, molecular functions and cellular components. (N=10 mice), two-sided FDR corrected Mann Whitney U ranksum test.

Extended Data Figure 12. NAM differentially expressed genes associated with Nuclear respiratory factor-1 (NRF-1). (a) Representation of spinal cord transcripts obtained by RNA-seq analysis that changed similarly after AM and NAM treatments of SOD1-Tg mice and share the GCGCMTGCGCN binding site for the Nuclear Respiratory Factor-1 (NRF-1) transcription factor. The analysis was done using the G:Profiler platform[28]. **(b)** Spinal cord global transcriptomic analysis in NAM or water treated SOD1-Tg mice and **(c)** individual representation of *Slc1a2*, (N=10). **(d)** Spinal cord global transcriptomic analysis in AM or PBS treated SOD1-Tg mice and **(e)** individual representation of *Slc1a2*, (N=7). Two-sided FDR-corrected Mann-Whitney ranksum test. Error bars represent mean ± S.E.M.

Extended Data Figure 13. Different gut microbiome composition in ALS patients. (a) Taxa summary representation at the species level of gut microbiome of healthy family members (N=29) and ALS patients (N=37) obtained by metagenomic shotgun sequencing. Two-sided FDR-corrected Mann-Whitney ranksum test. **(b)** Spearman correlation between *Bifidobacterium pseudocatenulatum* abundance obtained by metagenomic shotgun sequencing and serum NAM levels of ALS patients (N=37 ALS patients and 29 healthy controls). **(c)** KO relative abundances of microbiome-associated genes of the nicotinamide pathway in ALS (N=36) and healthy (N=28) stool samples. *q=0.03-0.04, two-sided FDR corrected Mann Whitney U ranksum test. Error bars represent mean ± S.E.M. **(d)** Top 97 differentially-represented serum metabolites between healthy individuals (N=13) and ALS patients (N=23) obtained by untargeted metabolomics. **(e)** Serum metabolites levels of tryptophan/nicotinamide pathways in ALS (N=24) patients and healthy individuals (N=13) obtained by non-targeted metabolomics. *P<0.05, **P<0.005, ***P<0.0005, two-sided FDR-corrected Mann-Whitney ranksum test. Error bars represent mean ± S.E.M. **(f)** Serum NAM correlation with clinical FRS scores and **(g)** bacterial L-aspartate oxidase copies in ALS patients (N=60) and healthy controls (N=33), ***P=0.0001, Mann Whitney U test and Spearman correlation coefficient.

Methods

Mice

Animal experiments were approved by the Weizmann Institute Animal Care and Use Committee Following US National Institute of Health, European Commission and the Israeli guidelines. G93A mSOD1-Tg mice on a C57BL/6 background were kindly provided by Eran Hornstein (Weizmann Institute). In all experiments, age- and gender-matched mice were used and WT littermates were used as controls. Mice were 40 days of age at the beginning of experiments. All mice were kept on a strict 24 hr reverse light-dark cycle, with lights being turned on from 10pm to 10am. For antibiotic treatment, mice were given a combination of vancomycin (0.5 g/l), ampicillin (1 g/l), neomycin (1 g/l), and metronidazole (1 g/l) in drinking water from age 40 days as previously described[29]. For the *Akkermansia muciniphila* or *Ruminococcus torques* colonization, 40 days old mice were treated with antibiotics for two weeks, followed by 2 days of washout period and gavaged with 200 µl of PBS-suspended bacteria (O.D.=0.7) weekly until the experimental end-point. Food intake and other metabolic parameters were measured using the PhenoMaster system (TSE-Systems, Bad Homburg, Germany), which consists of a combination of sensitive feeding sensors for automated measurement, and a photobeam-based activity monitoring system, which detects oxygen and carbon dioxide consumption, and records ambulatory movements, including rearing and climbing, in each cage. All parameters were measured continuously and

simultaneously. Mice were trained singly-housed in identical cages prior to data acquisition. All experimental procedures were approved by the local IACUC 02660418-3.

Administration of metabolites

For the *in vivo* administration of NAM and Phenol sulfate, the Alzet osmotic minipumps model 1004 (Charles River) were used (infusing the compound at a rate of 0.11 µL/hour for 4 weeks). The pumps were filled with 100 µL 50 mg/ml Nicotinamide (Cymit Quimica, Barcelona, Spain) or 33.33 mg/ml Phenol sulfate sodium salt (TLC, Ontario, Canada) diluted in sterile water (equivalent to 49.28 mg/kg/week of NAM and 30.8 mg/kg/week Phenol sulfate). Vehicle control pumps contained equivalent volume of Ultra-pure water. 6-week-old SOD1-Tg and WT litter-mate mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), the neck skin was shaved and sterilized with 70% ethanol, 1 cm incision was made in the skin, the osmotic minipumps were inserted following minimal blunt dissection and placed above the right hind flank. The cut was then closed with sterile surgical clips and the animals were carefully monitored for any signs of stress, bleeding, pain, or abnormal behavior. By replacing the pumps every 28 days, for a total of 4 times between the ages of 40-152 days, we assured steady and continuous metabolites administration to mice throughout the disease.

Assessment of motor functions in mice

Rotarod

To assess motor coordination and balance, each mouse was tested with a rotarod device (Panlab Le8500 Harvard Apparatus, Spain), in acceleration speed mode (increasing from 4 rpm to 40 rpm during 10 min), with a maximum test time of 5 min. The mice were habituated on the horizontal rotating rod and pre-trained for 3 trials before the formal tests. Each mouse was recorded three times at ages 60, 80, 100, 120 and 140 days. The apparatus automatically recorded the elapsed time when the mouse fell from the spindle.

Hanging wire grip test

Mice were allowed to grip a 2 mm thick horizontal metal wire (suspended 80 cm above the working surface) with their forepaws and the latency to successfully raise their hind legs to grip the wire was recorded. The mice were observed for 30 sec and scored as follows: 0 = falls off within 10 sec.; 1 = hangs onto bar by two forepaws; 2 = attempts to climb onto bar; 3 = hangs onto bar by two forepaws plus one or both hind paws; 4 = hangs by all four paws plus tail wrapped around bar; 5 = active escape to the end of bar.

Neurological scoring

Mice were neurologically scored by a system developed by ALS TDI [30]: Score of 0: Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds, suspended two to three times. Score of 1: Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension. Score of 2: Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table. Score of 3: Rigid paralysis or minimal joint movement, foot not being used for generating forward motion. Score of 4: Mouse cannot right itself within 30 sec after being placed on either side.

Home-cage locomotion

The locomotion of animals was quantified over a period of 46 h in the home cage, by automated sensing of body-heat image using an InfraMot (TSE-Systems). Individual animal movements were summed up every 30 min.

Survival

From age 130 days, mice were monitored daily. The endpoint was defined by reaching neurological score of 4 and/or more than 15% reduction in body weight. The probability of survival was calculated using the Kaplan– Meier method, and statistical analysis was performed using a log-rank test.

Cerebrospinal fluid (CSF) extraction

Mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The skin of the neck was shaved, and the mouse was placed prone on the stereotaxic instrument. The head was secured with head adaptors. The surgical site was swabbed with 70% ethanol, and a sagittal incision of the skin was made inferior to the occiput. Under the dissection microscope, the subcutaneous tissue and muscles (m. biventer cervicis and m. rectus capitis dorsalis major) were separated by blunt dissection with forceps. A pair of micro-retractors was used to hold the muscles apart. The dura mater was blotted dry with sterile cotton swab. CSF was collected using a capillary tube to penetrate into the cisterna magna (through the dura mater, lateral to the arteria dorsalis spinalis) and immediately frozen in liquid nitrogen and stored at -80^{o}C.

Magnetic resonance imaging (MRI)

During MRI scanning, mice were anesthetized with Isofluorane (5% for induction, 1–2% for maintenance) mixed with oxygen (1 liter/min) and delivered through a nasal mask. Once anesthetized, the animals were placed in a head-holder to assure reproducible positioning inside the magnet. Respiration rate was monitored and kept throughout the experimental period around 60–80 breaths per minute. MRI experiments were performed on 9.4 Tesla BioSpec Magnet 94/20 USR system (Bruker, Germany) equipped with gradient coil system capable of producing pulse gradient of up to 40 gauss/cm in each of the three directions. All MR images were acquired with a receive quadrature mouse head surface coil and transmitter linear coil (Bruker). The T_2 maps were acquired using the multi-slice spin-echo (MSME) imaging sequence with the following parameters: a repetition delay (TR) of 3000 ms, 16-time echo (TE) increments (linearly from 10 to 160ms), matrix dimension of 256 x 128 (interpolated to 256 x 256) and two averages, corresponding to an image acquisition time of 12 min 48 sec. The T_2 dataset consisted of 16 images per slice. Thirteen continuous slices with slice thickness of 1.00 mm were acquired with a field of view (FOV) of 2.0 x 2.0 cm^2.

Image Analysis

A quantitative T_2 map was produced from multi-echo T_2-weighted images. The multi-echo signal was fitted to a mono-exponential decay to extract the T_2 value for each image pixel. All image analysis was performed using homemade scripts written in Matlab R2013B. Co-registration inter-subject and intra-subject was applied before the MRI dataset analysis. For optimal suitability to a mouse brain atlas (correction of head movements image artifacts), all images went through atlas registration: reslicing, realignment and smoothing, using the SPM software (version 12, UCL, London, UK). The results were reported as mean ± SD. A t-test was used to compare means of two groups. A p value of less than 0.01 was considered statistically significant.

Histology

Sections from the spinal cord (C3-T6) were fixed in paraformaldehyde and embedded in paraffin for staining with luxol fast-blue and cresyl echt violet. Subsequently, sections were examined by a blinded researcher and cresyl echt violet positive motor neurons in the ventral horn were counted to evaluate neuronal survival. Colon tissues were fixed in dry methanolic-Carnoy and stained with the nuclear stain Sytox green and the Muc2 mucin with the anti-MUC2C3 antiserum and goat anti-rabbit-Alexa 555 (Thermo Fisher Scientific)[31]

Measuring gut epithelial barrier permeability by FITC-dextran

On the day of the assay, 4 kDa fluorescein isothiocyanate (FITC)-dextran was dissolved in PBS to a concentration of 80 mg ml^{-1}. Mice were fasted for 4 hours prior to gavage with 150µl dextran. Mice were anesthetized 3 hours following gavage and blood was collected and centrifuged at 1,000 x g for 12 min at 4°C. Serum was collected and fluorescence was quantified at an excitation wavelength of 485 nm and 535 nm emission wavelength.

Flow cytometry

WT and SOD1-Tg mice treated with Abx from 40 days of age or with water as controls were used for small-intestinal, colonic and spinal cord cellularity analysis either on day 140 (for small intestines and colons) or on days 60 and 140 (for spinal cords). Small intestinal and colonic samples were extensively washed from fecal matter followed by 2 mM EDTA dissociation in 37^{o}C for 30 min. Following extensive shaking, the epithelial fraction was discarded. Samples were then digested using DNaseI and collagenase for lamina propria analysis. Spinal cord samples were harvested from individual mice, homogenized and incubated with a HBSS solution containing 2% BSA (Sigma-Aldrich), 1 mg/ml collagenase D (Roche), and 0.15 mg/ml DNaseI, filtered through a 70 µm mesh. Homogenized sections were resuspended in 40% percoll, prior to density centrifugation (1000 × g. 15 min at 20°C with low acceleration and no brake). The isolated cells were washed with cold PBS and resuspended in PBS containing 1% BSA for direct cell surface staining. Single-cell suspensions were stained with antibodies for 45 min on ice against CD45, CD11b, CD11c, Ly6C, Ly6G, B220, CD3 and EpCAM (Biolegened). Stained cells were analyzed on a BD-LSRFortessa cytometer and were analyzed with FlowJo software.

Mucus proteomic analysis

For proteome analyses isolated mucus samples were incubated overnight at 37°C in reduction buffer (6M guanidinium hydrochloride, 0.1M Tris/HCl, pH 8.5, 5mM EDTA, 0.1 M DTT (Merck)) and soluble fraction was added on top of a spin-filter (10 kDa, PALL, Port Washington, NY) for a filter-aided sample preparation following a previous protocol[32] where 6M GuHCl was used instead of urea. Proteins on the filters were alkylated and subsequently digested for 4h with LysC (Wako, Richmond, VA) followed by an overnight trypsin (Promega, Fitchburg, WI) digestion. Heavy peptides (SpikeTides TQL, JPT Peptide Technologies, Berlin, Germany) for Muc2 absolute quantification (10 peptides, 100 fmol each[33] were added before trypsin digestion. Peptides released from the filter after centrifugation were cleaned with StageTip C18 columns[34]. NanoLC–MS/MS was performed on an EASY-nLC 1000 system (Thermo Fisher Scientific), connected to a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) through a nanoelectrospray ion source. Peptides were separated with an in-house packed reverse-phase

column (150 x 0.075 mm inner diameter, C18-AQ 3 µm) by a 30 min gradient from 10 to 45% of buffer B (A: 0.1% formic acid, B: 0.1% formic acid/80% acetonitrile) using a flow rate of 300 nl/min. Full mass spectra were acquired from 350–1,600 m/z with resolution of 60,000 (m/z 200). Up to 15 most intense peaks (charge state ≥ 2) were fragmented and tandem mass spectra were acquired with a resolution of 15,000 and 20 s dynamic exclusion. For absolute quantification a separate targeted mass spectrometry method was used where only precursors and their fragments of the heavy and corresponding light peptides were scanned with a resolution of 30,000. Proteins were identified with the MaxQuant program (version 1.5.7.4[35]) by searching against the mouse (downloaded 11.07.2018) UniProt protein database supplemented with an in-house database containing all the mouse mucin sequences (http://www.medkem.gu.se/mucinbiology/databases/). Searches were performed with full tryptic specificity, maximum 2 missed cleavages, precursor tolerance of 20 ppm in the first search used for recalibration, followed by 7 ppm for the main search and 0.5 Da for fragment ions. Carbamido-methylation of cysteine was set to fixed modification and methionine oxidation and protein N-terminal acetylation were set as variable modification. The required false discovery rate (FDR) was set to 1% both for peptide and protein levels and the minimum required peptide length was set to six amino acids. Proteins were quantified based on MaxQuant label-free quantification (LFQ) option using a minimum of two peptides for quantification. Absolute quantification of Muc2 was performed with Skyline (version 4.2.0[36]).

Bacterial cultures

Akkermansia muciniphila (ATCC BAA-835), *Akkermansia muciniphila* (ATCC BAA-2869), *Ruminococcus torques* (ATCC 27756), *Lactobacillus gasseri* (ATCC 33323), *Prevotella melaninogenica* (ATCC 25845), *Coprobacillus cateniformis* (DSM-15921), *Parabacteroides goldsteinii* (DSM-19448), *Lactobacillus murinus* (DSM-100194), *Parabacteroides distasonis* (ATCC 8503), *Eisenbergiella tayi* (DSM-24404) *Subdoligranulum variabile* (SDM-15176) were grown in chopped meat medium (BD 297307) under anaerobic conditions (Coy Laboratory Products, 75% N_2, 20% CO2, 5% H_2) in 37°C without shaking. *Eggerthella lenta* (DSM-15644) was grown in chopped meat medium supplemented with 0.5% arginine. All strains were validated for purity by whole-gene 16S sanger sequencing. WT and ΔnadA *E. coli* were kindly provided by Professor Shimshon Belkin (Institute of Life Sciences, the Hebrew University, Jerusalem, Israel), originally obtained from the "Keio collection[37]" and were grown on LB media (WT) or LB supplemented with 30 µg/ml kanamycin (ΔnadA). To measure bacterial *in-vitro* nicotinamide secretion, bacterial strains were grown in chopped meat medium until stationary phase, centrifuged and washed twice with M9 minimal medium with trace elements and glucose (4 g/l) and resuspended in M9 for 3 hrs under anaerobic conditions. Following centrifugation, 50 µl of the supernatant was collected for targeted nicotinamide measurements, and protein was extracted from the pellet using the BCA method: briefly: bacterial pellets were homogenized in RIPA buffer containing protease inhibitors, incubated for 45 min in 4 °C and centrifuged for 20 min, 14,000 r.p.m., at 4 °C. Nicotinamide measurement in the media were then normalized to the total protein level in each sample.

Nucleic acid extraction

DNA purification

Genomic DNA was purified using PowerMag Soil DNA isolation kit (Qiagen) optimized for Tecan automated platform. For shotgun sequencing, Illumina libraries were prepared using Nextera DNA Samp

Prep kit (Illumina, FC-121-1031), according to manufacturer's protocol and sequenced using the Illumina NextSeq platform with a read length of 80bp.

RNA Purification

Spinal cord samples were harvested from mice and snap-frozen in liquid nitrogen. Tissues were homogenized in Tri Reagent (Bio-Lab). RNA was purified using standard chloroform extraction. Two micrograms of total RNA were used to generate cDNA (HighCapacity cDNA Reverse Transcription kit; Applied Biosystems).

Real-time PCR was performed using Kapa Sybr qPCR kit (Kapa Biosystems) on a Viia7 instrument (Applied Biosystems). PCR conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Data were analyzed using the ΔΔCt method with 16S serving as the reference housekeeping gene. 16S cycles were assured to be insensitive to the experimental conditions.

Nucleic acid processing and library preparation

16S qPCR Protocol for Quantification of Bacterial DNA

DNA templates were diluted to 1 ng/µl before amplifications with the primer sets (indicated in Table I) using the Fast Sybr™Green Master Mix (ThermoFisher) in duplicates. Amplification conditions for *Akkermansia muciniphila* were: Denaturation 95°C for 3 minutes, followed by 40 cycles of denaturation 95°C for 3 seconds; annealing 66°C for 30 seconds followed by meting curve. Amplification conditions for total bacteria (16S rRNA) were: Denaturation 95°C for 3 minutes, followed by 40 cycles of denaturation 95°C for 3 seconds; annealing 60°C for 30 seconds followed by meting curve. Duplicates with >2 cycle difference were excluded from analysis. The CT value for any sample not amplified after 40 cycles was defined as 40 (threshold of detection).

16S rDNA Sequencing

For 16S amplicon pyrosequencing, PCR amplification was performed spanning the V3/4 region using the primers 515F/806R of the 16S rDNA gene and subsequently sequenced using 2x250 bp paired-end sequencing (Illumina MiSeq). Custom primers were added to Illumina MiSeq kit resulting in 253 bp fragment sequenced following paired end joining to a depth of 110,998 ± 66,946 reads (mean ± SD).

Read1: TATGGTAATTGTGTGCCAGCMGCCGCGGTAA

Read2: AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Index sequence primer: ATTAGAWACCCBDGTAGTCCGGCTGACTGACTATTAGAA

Whole genome shotgun sequencing

100 ng of purified DNA was sheared with a Covaris E220X sonicator. Illumina compatible libraries were prepared as described [38], and sequenced on the Illumina NextSeq platform with a read length of 80bp to a depth of 10M reads for human samples, 1M reads for AM treated mice samples and 5M reads for the comparison between naïve WT and SOD1-Tg mice.

RNA-Seq

Ribosomal RNA was selectively depleted by RNaseH (New England Biolabs, M0297) according to a modified version of a published method [39]. Specifically, a pool of 50bp DNA oligos (25 nM, IDT, indicated in Table II) that is complementary to murine rRNA18S and 28S, was resuspended in 75 µl of 10 mM Tris

pH 8.0. Total RNA (100-1000 ng in 10 µl H_2O) were mixed with an equal amount of rRNA oligo pool, diluted to 2 µl and 3 µl 5x rRNA hybridization buffer (0.5 M Tris-HCl, 1 M NaCl, titrated with HCl to pH 7.4) was added. Samples were incubated at 95°C for 2 minutes, then the temperature was slowly decreased (−0.1°C/s) to 37°C. RNaseH enzyme mix (2 µl of 10U RNaseH, 2 µl 10 x RNaseH buffer, 1 µl H_2O, total 5 µl mix) was prepared 5 minutes before the end of the hybridization and preheated to 37°C. The enzyme mix was added to the samples when they reached 37°C and they were incubated at this temperature for 30 minutes. Samples were purified with 2.2x SPRI beads (AMPure XP, Beckmann Coulter) according to the manufacturers' instructions. Residual oligos were removed with DNase treatment (ThermoFisher Scientific, AM2238) by incubation with 5 µl DNase reaction mix (1 µl Trubo DNase, 2.5 µl Turbo DNase 10 x buffer, 1.5 µl H_2O) that was incubated at 37°C for 30 minutes. Samples were again purified with 2.2x SPRI beads and suspended in 3.6 µl priming mix (0.3 µl random primers of New England BioLabs, E7420, 3.3 µl H_2O). Samples were subsequently primed at 65°C for 5 minutes. Samples were then transferred to ice and 2 µl of the first strand mix was added for first strand cDNA synthesis (1 µl 5x first strand buffer, NEB E7420; 0.125 µl RNase inhibitor, NEB E7420; 0.25 µl ProtoScript II reverse transcriptase, NEB E7420; and 0.625 µl of 0.2 µl/ml Actinomycin D, Sigma, A1410). The first strand synthesis and all subsequent library preparation steps were performed using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, E7420) according to the manufacturers' instructions (all reaction volumes reduced to a quarter).

16S rDNA analysis

Overlapping paired-end FASTQ files were matched and processed in a data curation pipeline implemented in Qiime2 version 2018.4.0 (Qiime2)[40]. Paired-end sequence data were demultiplexed according to sample specific barcodes using Qiime2 demux-emp-paired. Trimming and amplicon sequence variant (ASV) picking were carried out with the use of DADA2[41]. Alpha rarefaction curves were plotted using Qiime2 alpha-rarefaction and were used to set an appropriate subsampling depth for each comparison. Samples were rarefied using Qiime2 feature-table rarefy[42]. Samples with a read depth lower than the relevant subsampling depth were excluded from the analysis. ASV's were assigned with taxonomic annotations using a Naïve-Bayes fitted classifier trained on August 2013, 97% identity Greengenes rRNA database[43]. Relative abundance tables were calculated using Qiime2 feature-table summarize-taxa. Ordination plots were calculated from Unweighted and Weighted UniFrac distance matrix using principal coordinates analysis (PCoA).

Metagenomic analysis

For metagenome analysis, metagenomic reads containing Illumina adapters and low-quality reads were filtered and low-quality read edges were trimmed. Host DNA was detected by mapping with GEM [44] to the human or mouse genome (hg19 or mm10 respectively) with inclusive parameters, and host reads were removed. For SOD1-Tg vs. WT naïve mice metagenomes 5 million reads were subsampled, 1 million reads for AM-treated mice and for humans 7-10 million reads. Relative abundances from metagenomic sequencing were computed using MetaPhlAn2[45] with default parameters. MetaPhlAn relative abundances were capped at a level of $5x10^{-4}$. KO relative abundance was obtained by mapping to KEGG [46] bacterial genes database using DIAMOND [47], considering only the first hit, and allowing e-value < 0.0001. The relative abundance of a KO was determined as the sum of all reads mapped to bacterial genes associated with that KO, divided by the total number of mapped reads in a sample. For mice samples, KO relative abundance was obtained by first mapping to IGC, an integrated catalog of reference genes in the human

gut microbiome, and then summing the relative abundance of all the genes in each KO to obtain relative abundance. KO relative abundances were capped at a level of $2x10^{-5}$ for mice and $2x10^{-7}$ for humans. Taxa and KOs present in less than 10% of samples were discarded.

Metabolites selection

Using the top 12 significant serum metabolites altered by Abx in WT and SOD1-Tg mice, we first downloaded all nucleotide sequences of KEGG genes with potential to synthesize or degrade the 12 metabolites. Next, we built a bowtie index of KEGG genes and mapped to it SOD1-Tg and WT metagenome samples. Finally, we obtained all mapped reads and for every sample and KEGG gene, we report the number of reads mapped to the KEGG gene and its mean score. Scores are as defined by bowtie2[48] and range between 0 to -45, where 0 denotes perfect match.

RNAseq analysis

Data pre-processing

bcl files were converted to fastq and adaptor trimming was performed using bcl2fastq. Then, reads were aligned to the mm10 reference genome (UCSC) using STAR (splice site aware alignment). Secondary alignments and PCR/optical duplicates were removed using samtools view -h -F 256 -F 1024. Alignments were binned to genes using htseq-count (htseq-count -a 5 -s reverse -r). Transcript integrity number (TIN) medians were calculated using RSeQC. (tin.py.bed file: mm10 RefSeq.bed.gz downloaded from https://sourceforge.net/projects/rseqc/files/BED/Mouse_Mus_musculus/)

Differential gene expression

For each comparison, genes with reads ≥ 10^{-4} out of total reads and expressed in at least fifth of a group in each comparison were included in the analysis. Deseq2 models were fitted for each comparison separately [design: counts ~ group + median (TIN)]. Differentially expressed genes were found using Wald-test on Deseq2 objects. Heatmaps were created using the regularized log transformed data (rlog).

gene set enrichment analysis

For each gene, we calculated the following score out of its DESeq results: -log(padj} sign(log2FoldChange). bulk.gsea function was used from liger package, with the http://ge-lab.org/gskb/2-MousePath/MousePath_GO_gmt.gmt as our universe model.

Non-targeted metabolomics

Sera and cecal samples were collected, immediately frozen in liquid nitrogen and stored at -80^{o}C. Sample preparation and analysis was performed by Metabolon Inc. Samples were prepared using the automated MicroLab STAR system (Hamilton). To remove protein, dissociated small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol. The resulted extract was divided into five fractions: one for analysis by UPLC-MS/MS with negative ion mode electrospray ionization, one for analysis by UPLC-MS/MS with positive ion mode electrospray ionization, one for LC polar platform, one for analysis by GC-MS and one sample was reserved for backup. Samples were placed briefly on a TurboVap (Zymark) to remove the organic solvent. For LC, the samples were stored overnight under nitrogen before preparation for analysis. For GC, each sample was dried under vacuum overnight before preparation for analysis.

Data extraction and compound identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Compound were identified by comparison to library entries of purified standards or recurrent unknown entities.

Metabolite quantification and data normalization

Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences.

Targeted metabolomics

50ng/ml of D4-Nicotinamide (Cambridge Isotope Laboratories) were added to all samples as internal standards. The samples (in 50% Methanol) were dried in a speed vac to blow off the methanol before drying to completion in a lyophilizer. All samples were re-dissolved in 100 µl of 0.1% formic acid.

Liquid Chromatography

Liquid chromatography was performed on a Waters Acquity UPLC system. Metabolites were separated on an Acquity HSS T3 column (2.1 × 150 mm, 1.8 µm particle size; Waters) at 40°C using a 10-min program. Mobile phase consisted of (A) water and (B) acetonitrile each containing 0.1% formic acid. Gradient conditions were: 0 to 1 min = 99.9% A 0.1% B; 1 to 6 min = 0.1% to 10.0% B; 6 to 7 min = 10% to 100% B; 7.0 to 7.2 min = 100% B; 7.2 to 10 min = 99.9% A, 0.1% B. Injection volume was 1.0 µl, and flow rate was 0.3 ml/min.

Mass Spectrometry

LC-MS/MS analysis was performed on a Waters Xevo triple quadrupole equipped with a Zspray ESI source. MRM was performed in the positive ion mode. Other MS parameters included: desolvation temperature at 600°C, desolvation gas flow at 900L/Hr, cone gas flow at 150L/Hr nebulizer pressure at 7 Bar, capillary voltage (CV) at 2.53kV. The MRM transitions used were: (a) Nicotinamide: 123 > 78 and 123 > 80, CE 19, 13 V respectively and (b) D4-Nicotinamide 127 > 81 and 127 > 84, CE 19, 17 V respectively. Argon (0.10 mg/min) was used as collision gas. TargetLynx (Waters) was used for Qualitative and Quantitative analysis.

Patients and control individuals

Human observational study

The human observational study was approved by the Hadassah Medical Center Institutional Review Board (IRB approval numbers HMO-16-0396) and Weizmann Institute of Science Bioethics and Embryonic Stem Cell Research oversight committee (IRB approval numbers 365-1). Written informed consent was obtained from all subjects.

Exclusion and inclusion criteria (human observational study)

All subjects fulfilled the following inclusion criteria: males and females, aged 18-70, who are currently not following any interventional diet modification other than advice to avoid nutritional deficiencies and are able to provide informed consent. Exclusion criteria included: (i) pregnancy or fertility treatments; (ii) usage of antibiotics or antifungals within three months prior to participation; (iii) consumption of probiotics in any form within one month prior to participation, (iv) active inflammatory or neoplastic disease three years prior to enrollment; (v) chronic gastrointestinal disorder, including inflammatory bowel disease and celiac disease; (vi) myocardial infarction or cerebrovascular accident in the 6 months prior to participation; (vii) coagulation disorders; (viii) chronic immunosuppressive medication usage; (ix)

pre-diagnosed type I or type II diabetes mellitus or treatment with anti-diabetic medication. None of the enrolled patients and controls reported of constipation or on taking constipation medications. One of the ALS patients had a chronic diagnosis of irritable bowel syndrome (IBS), another was diagnosed with asymptomatic PBC for 8 years prior to development of ALS, and was consuming Ursodecholic acid (1500mg per day). Two patients were diagnosed with chronic dyspepsia and one with reflux esophagitis. Two of them were chronically treated (for years) with proton pumps inhibitors. Adherence to inclusion and exclusion criteria was validated by medical doctors.

Statistical Analysis

Data are expressed as mean ± SEM. p values < 0.05 were considered significant ($*p < 0.05$; $**p < 0.05$; $***p < 0.005$; $****p < 0.0005$). Pairwise comparisons were performed using Student's t test. Mann-Whitney U test was used when the distribution was not known to be normal. Comparison between multiple groups was performed using ANOVA, and FDR correction was used to adjust for multiple comparisons. We analyzed the effect of Abx over time in control and SOD1-Tg mice by modeling neuro-phenotypical measurements (rotarod, grip test score and neurological score) as a function of time and treatment in a time-depended manner using a linear regression:

Phenotype ~ time + time x treatment + time x genotype + time x treatment x genotype

where time is the day (60, 80, 100, 120 and 140), treatment (± Abx) and genotype (WT or SOD1-Tg) are binary indicators. Significance of treatment is then inferred by the p-value of the time x treatment predictor. For this analysis we used python statsmodels.api.ols version 0.8.0 statsmodels.

Microbial abundance change over time was evaluated using linear regression:

OTU ~ time + time x genotype

The significance of genotype effecting OTU abundance was inferred by the p-value of the time x genotype predictor after 5% FDR correction for multiple OTUs.

To analyze KOs of the nicotinamide and tryptophan metabolic pathways KO levels between groups were compared using Mann Whitney U ranksum test. For this analysis we use python stats.HigherLevelRanksum.directed_mannwhitneyu.